

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/82, 5/10, 5/14, 15/00, 15/09, 15/29, 15/32, A01H 1/00, 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/05261</b> <b>(43) International Publication Date:</b> 13 February 1997 (13.02.97)
<b>(21) International Application Number:</b> PCT/US96/12158 <b>(22) International Filing Date:</b> 24 July 1996 (24.07.96) <b>(30) Priority Data:</b> 08/508,786 28 July 1995 (28.07.95) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 08/508,786 (CON) Filed on 28 July 1995 (28.07.95) <b>(71) Applicant (for all designated States except US):</b> NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holladay Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CONKLING, Mark, A. [US/US]; 5313 April Wind Drive, Fuquay-Varina, NC 27526 (US). MENDU, Nandini [IN/US]; 5639 Chapel Hill Road 207, Durham, NC 27707 (US). SONG, Wen [CN/US]; 2702 Vanderbilt Avenue, Raleigh, NC 27607 (US). <b>(74) Agents:</b> SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).	<b>(81) Designated States:</b> AL, AM, AT, AT (Utility model), AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> ROOT CORTEX SPECIFIC GENE PROMOTER  <b>(57) Abstract</b>  An isolated DNA molecule comprises a DNA promoter sequence which directs root cortex specific transcription of a downstream heterologous DNA segment in a plant cell. A DNA construct comprises an expression cassette comprising, in the 5' to 3' direction, a promoter of the present invention and a heterologous DNA segment positioned downstream from the promoter and operatively associated therewith. Transformed plants, such as tobacco plants, comprise transformed plant cells containing a heterologous DNA construct comprising an expression cassette as described above.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Larvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

-1-

### ROOT CORTEX SPECIFIC GENE PROMOTER

This invention was made with government support under Grant No. MCB-9206506 from the National Science Foundation. The government may have certain rights to this invention.

#### 5. Field of the Invention

This invention relates to tissue-specific gene promoters, and particularly relates to a promoter which is active in the root cortex of plants.

#### Background of the Invention

10 A promoter is a DNA sequence which flanks a transcribed gene, and to which RNA polymerase must bind if it is to transcribe the flanking gene into messenger RNA. A promoter may consist of a number of different regulatory elements which affect a structural gene  
15 operationally associated with the promoter in different ways. For example, a regulatory gene may enhance or repress expression of an associated structural gene, subject that gene to developmental regulation, or contribute to the tissue-specific regulation of that  
20 gene. Modifications to promoters can make possible optional patterns of gene expression, using recombinant DNA procedures. See, e.g., Old and Primrose, Principles of Gene Manipulation (4th Ed., 1989).

One example of a plant promoter is the promoter  
25 found flanking the gene for the small subunit ribulose-1,5-bisphosphate carboxylase in Petunia. See U.S. Patent No. 4,962,028. Another example is the promoter which comprises the 5' flanking region of the wheat Em gene. See EPO Appln. No. 335528. Still another example is the  
30 stress-inducible regulatory element disclosed in EPO Appln. No. 0 330 479.

-2-

Despite their important role in plant development, relatively little work has been done on the regulation of gene expression in roots. In part the deficiency results from a paucity of readily  
5 identifiable, root-specific biochemical functions whose genes may be easily cloned and studied. Evans et al., *Mol. Gen. Genet.* 214, 153-157 (1988), tried unsuccessfully to isolate root-specific cDNA clones from pea, concluding that root-specific mRNA species (if  
10 present) are only present at a very low level of abundance in the root mRNA population. Fuller et al., *Proc. Natl. Acad. Sci. USA* 80, 2594-2598 (1983), have cloned and characterized a number of root nodule-specific genes. Comparisons of the DNA sequences 5' of the  
15 initiation of transcription reveal a repeated octanucleotide present in the three genes examined. Unfortunately, the lack of efficient transformation/regeneration systems for most *Leguminaceae* has hampered the functional analysis of such *cis*-acting  
20 sequences. Bogusz et al., *Nature* 331, 178-180 (1988), isolated a haemoglobin gene expressed specifically in roots of non-nodulating plants by its homology with the haemoglobin gene of closely related, nodulating species. Keller and Lamb, *Genes & Dev.* 3, 1639-1646 (1989),  
25 isolated a gene encoding a cell wall hydroxyproline rich glycoprotein expressed during lateral root initiation. Lerner and Raikhel, *Plant Physiol.* 91, 124-129 (1989), recently reported the cloning and characterization of a barley root-specific lectin.

30 Many plant pathogens and pests damage plant roots, causing serious crop damage and loss. The root tissue most often damaged is the root cortex, a layer composed primarily of storage parenchyma which underlies the epidermis layer and surrounds the central vascular  
35 cylinder of the root. The root cortex may additionally contain schlerenchyma, secretory cells, resin ducts and other structures and cells types. The cells of the root

-3-

cortex exhibit morphological and developmental similarities with cortical cells of the aerial shoot.

To impart useful traits to plants by the expression of foreign genes using genetic engineering techniques, a variety of tissue-specific promoters will be required to allow new traits to be expressed selectively in the appropriate plant tissues. The present invention is based upon our continuing investigations in connection with this problem.

10                   Summary of the Invention

The present invention is based on the identification of the tobacco RD2 (TobRD2) promoter, which directs root cortex specific expression of associated genes. A first aspect of the present invention is an isolated DNA molecule which directs root cortex specific transcription of a downstream heterologous DNA segment in a plant cell, the isolated DNA molecule having a sequence selected from the group consisting of (a) SEQ ID NOS:1-9 provided herein, and (b) DNA sequences which hybridize to any of SEQ ID NOS:1-9 under stringent conditions, and which direct root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

A further aspect of the present invention is an expression cassette comprising a Tobacco RD2 promoter and a heterologous DNA segment positioned downstream from, and operatively associated with, the promoter.

A further aspect of the present invention is an expression cassette comprising a root cortex specific promoter and a heterologous DNA segment, the sequence of the root cortex specific promoter selected from SEQ ID NOS:1-9 provided herein, and DNA sequences which hybridize to any of SEQ ID NOS:1-9 under stringent conditions, and which directs root cortex specific transcription.

-4-

Further aspects of the present invention are plant cells containing the above described expression cassettes, methods of making transformed plants from such plant cells, and the transformed plants comprising such  
5 transformed plant cells.

#### Brief Description of the Drawings

Figure 1A shows *in situ* localization of Tobacco RD2 transcripts in a transverse section of tobacco root from a seven day old seedling.

10        Figure 1B shows *in situ* localization of Tobacco RD2 transcripts in a longitudinal section of tobacco root from a seven day old seedling.

Figure 2 is a 2010 base pair sequence (SEQ ID NO:1) of the 5' region of TobRD2.

15        Figure 3 is a schematic showing the TobRD2 promoter/glucurodinase (GUS) constructs used to test the ability of the RD2 promoter to direct root cortex specific gene expression.

Figure 4 is a bar graph summarizing  $\beta$ -  
20 glucurodinase (GUS) activity in roots (solid bars), leaves (stippled bars) and stems (dotted bars) of plants transformed with chimeric reporter gene constructs, as provided in Table 1. The graph shows activity among plants transformed with gene constructs utilizing  
25 different promoters (CaMV35S;  $\Delta$ 2.00;  $\Delta$ 1.50;  $\Delta$ 1.40;  $\Delta$ 1.25;  $\Delta$ 0.80;  $\Delta$ 0.70;  $\Delta$ 0.60;  $\Delta$ 0.30) and utilizing the vector pBI101.3 alone as a control. GUS activity was measured in pmolMU/ $\mu$ g protein/min.

Figure 5A is a bar graph summarizing the  
30 relative  $\beta$ -glucurodinase (GUS) activity in roots and leaves of tobacco plants transformed with chimeric reporter gene constructs using different promoters (CaMV35S;  $\Delta$ 2.00;  $\Delta$ 1.50;  $\Delta$ 1.40;  $\Delta$ 1.25;  $\Delta$ 0.80;  $\Delta$ 0.70;  $\Delta$ 0.60;  $\Delta$ 0.30) and utilizing the vector pBI101.3 alone as  
35 a control, as provided in Table 1. GUS activity was

-5-

measured in pmolMU/ $\mu$ g protein/min, and the relative activity shown is root activity/leaf activity.

Figure 5B is a bar graph summarizing the relative  $\beta$ -glucuronidase (GUS) activity in roots and stems of plants transformed with chimeric reporter gene constructs using different promoters (CaMV35S;  $\Delta$ 2.00;  $\Delta$ 1.50;  $\Delta$ 1.40;  $\Delta$ 1.25;  $\Delta$ 0.80;  $\Delta$ 0.70;  $\Delta$ 0.60;  $\Delta$ 0.30) and utilizing the vector pBI101.3 alone as a control, as provided in Table 1. GUS activity was measured in pmolMU/ $\mu$ g protein/min, and the relative activity shown is root activity/stem activity.

Figure 6A is a photomicrograph showing the histochemical localization of GUS activity in a transverse section of root from a tobacco plant transformed with a reporter gene (GUS) driven by the  $\Delta$ 2.0 promoter.

Figure 6B is a photomicrograph showing the histochemical localization of GUS activity in a root tip from a tobacco plant transformed with a reporter gene (GUS) driven by the  $\Delta$ 2.0 promoter.

#### Detailed Description of the Invention

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

Transgenic plants expressing peptides that inhibit or kill a particular pest or pathogen provide a method for decreasing crop damage and loss. For example, expression of the *Bacillus thuringiensis* protein in transgenic corn provides resistance to the European corn bore. However, transgene expression in all tissues of a plant (constitutive expression) is disadvantageous as it can expose non-target organisms to the transgenic protein and in addition increases the selective pressure for the development of pathogens and pests which are resistant to



-6-

the transgenic protein. High levels of transgene expression throughout a plant may also negatively affect growth and yield of the plant. An alternative strategy is to express a toxic peptide only in the organ or tissue  
5 affected by a particular pest or pathogen. Implementation of this strategy against pests and pathogens that attack plant roots has been hampered by the lack of characterized root-specific promoters.

Transcription of a gene is initiated when a  
10 stable complex is formed between RNA polymerase enzyme and a gene promoter. Promoters occur at the beginning of all transcription units, are typically about 100 base pairs in length, and are located immediately upstream from the start site of transcription. See e.g., Maniatis  
15 et al., *Science* 236:1238 (1987). Promoters vary in their 'strength', that is, in their ability to accurately and efficiently initiate transcription. The RNA polymerase holoenzyme is thought to cover a region of about 50 bases immediately upstream of the transcribed region. In some  
20 cases the strength of transcription initiation may be enhanced by auxiliary proteins that bind adjacent to the region of the promoter which is immediately upstream from the transcribed DNA. See, e.g., Singer & Berg, Genes and Genomes, 140-145, University Science Books, Mill Valley,  
25 CA (1991).

Specific examples of root cortex specific promoters of the present invention are DNA molecules which have a sequence corresponding to any one of those shown in SEQ ID NOS: 1-9, all of which are discussed in  
30 greater detail below. It will be apparent that other sequence fragments from the Tobacco RD2 5' flanking region, longer or shorter than the foregoing sequences, or with minor additions, deletions, or substitutions made thereto, can be prepared which will also carry the TobRD2  
35 root cortex specific promoter, all of which are included within the present invention. A further aspect of the present invention includes promoters isolated from other

-7-

tobacco genes, or from plants other than tobacco as set forth below, which are homologous to the tobacco RD2 promoter and are capable of directing root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

As used herein, a TobRD2 promoter refers to a DNA molecule having a sequence identical to, or substantially homologous to, a continuous segment of the DNA found 5' to the transcribed region of the tobacco RD2 gene. SEQ ID NO:1 given herein provides the sequence of the 2 kb region found immediately 5' to the initiation of transcription in the TobRD2 gene. TobRD2 promoters include the at least the 100 base pair region, the 150 base pair region, or preferably the 200 base pair region immediately 5' to the TobRD2 transcribed region, and direct root cortex specific expression. As used herein, regions that are 'substantially homologous' are at least 75%, and more preferably are 80%, 85%, 90% or even 95% homologous.

As used herein, a root cortex specific promoter is a promoter that preferentially directs expression of an operatively associated gene in root cortex tissue, as compared to expression in leaf or stem tissue, or other tissues of the root.

Root cortex specific promoter sequences from other plants include those which are at least about 75 percent homologous (and more preferably 80%, 85%, 90% or even 95% homologous) to the approximately 100 base segment of the Tobacco RD2 promoter immediately upstream of the transcribed DNA region, and which are capable of directing root cortex specific transcription of a downstream heterologous DNA segment in a plant cell. Root cortex specific promoters from other plants include those which are at least about 75 percent homologous (and more preferably 80%, 85%, 90% or even 95% homologous) to the continuous portions of the TobRD2 promoter as defined herein by SEQ ID NOS: 1-9, and which are capable of

-8-

directing root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

High stringency hybridization conditions which will permit homologous DNA sequences to hybridize to a DNA sequence as given herein are well known in the art. For example, hybridization of such sequences to DNA disclosed herein may be carried out in 25% formamide, 5X SSC, 5X Denhardt's solution, with 100  $\mu$ g/ml of single stranded DNA and 5% dextran sulfate at 42°C, with wash conditions of 25% formamide, 5X SSC, 0.1% SDS at 42°C for 15 minutes, to allow hybridization of sequences of about 60% homology. More stringent conditions are represented by a wash stringency of 0.3M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60° or even 70°C using a standard *in situ* hybridization assay. (See Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory)). In general, plant DNA sequences which code for root cortex specific promoters and which hybridize to the DNA sequence encoding the tobacco RD2 root cortex specific promoters disclosed herein will be at least 75%, 80%, 85%, 90% or even 95% homologous or more with the sequences of the DNA encoding the tobacco RD2 root cortex specific promoters disclosed herein.

Root cortex specific promoters of the present invention are useful in directing tissue specific expression of transgenes in transformed plants. Such tissue-specific transgene expression is useful in providing resistance against damage caused by pests and pathogens which attack plant roots. In addition, as the root cortex is a major sink organ for photosynthate storage, expression of transgenes designed to alter the stored carbohydrates may be directed by such promoters. Exogenous genes of particular interest for root-cortex specific expression include those that code for proteins that bind heavy metals (such as metallothionein); proteins that give resistance to soil borne pests and pathogens; proteins that confer resistance to heat, salt

-9-

(salinity) and drought; proteins for desalinization; and proteins that metabolize plant storage compounds into alternative preferred products or forms.

Tissue specific promoters may also be used to  
5 convert pro-pesticides to active forms in selected tissue sites. Hsu et al. *Pestic. Sci.*, 44, 9 (1995) report the use of a chimeric gene comprising the root-specific promoter TobRB7 and the  $\beta$ -glucuronidase enzyme gene, to preferentially convert a pro-pesticide to an active form  
10 in roots. The inactive pro-pesticide (a glucuronide of hydroxymethyloxamyl) was applied to foliage and was then transported through plant phloem to roots, where it was converted to an active nematocidal form by glucuronidase.

Additionally, root-cortex specific promoters  
15 are useful for histological purposes, to identify or stain root-cortex tissue using a reporter gene such as  $\beta$ -glucurodinase.

The term "operatively associated," as used herein, refers to DNA sequences contained within a single  
20 DNA molecule which are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a gene when it is capable of affecting the expression of that gene (i.e., the gene is under the transcriptional control of the promoter). The  
25 promoter is said to be "upstream" from the gene, which is in turn said to be "downstream" from the promoter.

DNA constructs, or "expression cassettes," of the present invention include, 5'-3' in the direction of transcription, a promoter of the present invention, a  
30 heterologous DNA segment operatively associated with the promoter, and, optionally, transcriptional and translational termination regions such as a termination signal and a polyadenylation region. All of these regulatory regions should be capable of operating in the  
35 transformed cells. The 3' termination region may be derived from the same gene as the transcriptional initiation region or from a different gene.

-10-

Plants may be divided into those lacking chlorophyll (such as fungi) and those containing chlorophyll (such as green algae, mosses); and further divided into those containing chlorophyll and having  
5 vascular tissue (such as ferns, gymnosperms, conifers, monocots and dicots). The latter group of plants includes those in which roots, stems and leaves may be present. As used herein, the term 'plant' encompasses all such organisms described above. As used herein, the  
10 term 'natural plant DNA' means DNA isolated from non-genetically altered, or untransformed, plants (for example, plant varieties which are produced by selective breeding).

As used herein, the term heterologous gene or  
15 heterologous DNA segment means a gene (or DNA segment) which is used to transform a cell by genetic engineering techniques, and which may not occur naturally in the cell. Structural genes are those portions of genes which comprise a DNA segment coding for a protein, polypeptide,  
20 or portion thereof, possibly including a ribosome binding site and/or a translational start codon, but lacking a promoter. The term can also refer to copies of a structural gene naturally found within a cell but artificially introduced. Structural genes may encode a  
25 protein not normally found in the plant cell in which the gene is introduced or in combination with the promoter to which it is operationally associated. Genes which may be operationally associated with a promoter of the present invention for expression in a plant species may be  
30 derived from a chromosomal gene, cDNA, a synthetic gene, or combinations thereof. As used herein, the term heterologous DNA segment also includes DNA segments coding for non-protein products, such as ribozymes or anti-sense RNAs. Antisense RNAs are well known (see,  
35 e.g., US Patent No. 4,801,540 (Calgene, Inc.)).

Genes of interest for use with the present invention in plants include those affecting a wide

-11-

variety of phenotypic and non-phenotypic properties. Among the phenotypic properties are proteins, such as enzymes, which provide resistance to various environmental stresses, including but not limited to stress caused by dehydration (resulting from heat, salinity or drought), herbicides, toxic metals, trace elements, pests and pathogens. Resistance may be due to a change in the target site, enhancement of the amount of a target protein in the host cell, increased amounts of one or more enzymes involved with the biosynthetic pathway of a product which protects the host against the stress, and the like. Structural genes may be obtained from prokaryotes or eukaryotes, bacteria, fungi, (e.g., from yeast, viruses, plants, and mammals) or may be synthesized in whole or in part. Illustrative genes include glyphosphate resistant 3-enolpyruvylphosphoshikinate synthase gene, nitrilase, genes in the proline and glutamine biosynthetic pathway, and metallothioneins.

Structural genes operatively associated with the promoter of the present invention may be those which code for a protein toxic to insects, such as a *Bacillus thuringiensis* crystal protein toxic to insects. A DNA sequence encoding a *B. thuringiensis* toxin toxic to Coleoptera, and variations of this sequence wherein the coded-for toxicity is retained, is disclosed in U.S. Patent No. 4,853,331 (see also U.S. Patents Nos. 4,918,006 and 4,910,136) (the disclosures of all U.S. Patent references cited herein are to be incorporated herein in their entirety by reference). A gene sequence from *B. thuringiensis* which renders plant species toxic to Lepidoptera is disclosed in PCT Application WO 90/02804. PCT Application WO 89/04868 discloses transgenic plants transformed with a vector which promotes the expression of a *B. thuringiensis* crystal protein, the sequence of which may be employed in connection with the present invention. PCT Application

-12-

WO 90/06999 discloses DNA encoding a *B. thuringiensis* crystal protein toxin active against Lepidoptera. Another gene sequence encoding an insecticidal crystal protein is disclosed in U.S. Patent No. 4,918,006.

5 Exemplary of gene sequences encoding other insect toxins are gene sequences encoding a chitinase (e.g., EC-3.2.1.14), as disclosed in U.S. Patent No. 4,940,840 and PCT Appln. No. WO 90/07001. A gene coding for a nematode-inducible pore protein useful in producing

10 transgenic plants resistant to root nematodes is disclosed in U.S. Patent Application No. 08/007,998. Strains of *B. thuringiensis* which produce polypeptide toxins active against nematodes are disclosed in U.S. Patents Nos. 4,948,734 and 5,093,120 (Edwards et al.).

15 Where the expression product of the gene is to be located in a cellular compartment other than the cytoplasm, the structural gene may be constructed to include regions which code for particular amino acid sequences which result in translocation of the product to

20 a particular site, such as the cell plasma membrane, or secretion into the periplasmic space or into the external environment of the cell. Various secretory leaders, membrane integration sequences, and translocation sequences for directing the peptide expression product to

25 a particular site are described in the literature. See, for example, Cashmore et al., *Biotechnology* (1985) 3:803-808, Wickner and Lodish, *Science* (1985) 230:400-407.

The expression cassette may be provided in a

30 DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli*, such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting

35 construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range

-13-

replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there may be at least one marker present, which may be  
5 useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may provide  
10 protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation by imparting prototrophy to an auxotrophic host; or may provide a visible phenotype through the production of a novel compound in the plant.  
15 Exemplary genes which may be employed include neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT), nitrilase, and the gentamicin resistance gene. For plant host selection, non-limiting examples of suitable markers  
20 are beta-glucuronidase (GUS) (providing indigo production), luciferase (providing visible light production), NPTII (providing kanamycin resistance or G418 resistance), HPT (providing hygromycin resistance), and the mutated *aroA* gene (providing glyphosate  
25 resistance).

The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system and  
30 insertion of the particular construct or fragment into the available site. After ligation and cloning, the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature. See, e.g., Maniatis et al., Molecular  
35 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982).



-14-

A vector is a replicable DNA construct. Vectors which may be used to transform plant tissue with DNA constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as  
5 vectors suitable for DNA-mediated transformation. *Agrobacterium tumefaciens* cells containing a DNA construct of the present invention, wherein the DNA construct comprises a Ti plasmid, are useful in methods of making transformed plants. Plant cells are infected  
10 with an *Agrobacterium tumefaciens* to produce a transformed plant cell, and then a plant is regenerated from the transformed plant cell.

Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For  
15 example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium* vector is disclosed in U.S. Patent No. 4,795,855.  
20 Further, U.S. Patent No. 4,940,838 to Schilperoort et al. discloses a binary *Agrobacterium* vector (i.e., one in which the *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T-DNA region, and a second plasmid having a T-DNA region but no vir region)  
25 useful in carrying out the present invention.

Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present  
30 invention. The microparticle is propelled into a plant cell to produce a transformed plant cell and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present  
35 invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Agracetus European Patent Application Publication

-15-

No. 0 270 356, titled "Pollen-mediated Plant Transformation". When using ballistic transformation procedures, the expression cassette may be incorporated into a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

10 A transformed host cell is a cell which has been transformed or transfected with constructs containing a DNA sequence as disclosed herein using recombinant DNA techniques. Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

The promoter sequences disclosed herein may be used to express a heterologous DNA sequence in any plant species capable of utilizing the promoter (i.e., any plant species the RNA polymerase of which binds to the promoter sequences disclosed herein). Examples of plant species suitable for transformation with the DNA constructs of the present invention include both monocots and dicots, and include but are not limited to tobacco, soybean, potato, cotton, sugarbeet, sunflower, carrot, celery, flax, cabbage and other cruciferous plants, pepper, tomato, citrus trees, bean, strawberry, lettuce, maize, alfalfa, oat, wheat, rice, barley, sorghum and canola. Thus an illustrative category of plants which may be transformed with the DNA constructs of the present invention are the dicots, and a more particular category of plants which may be transformed using the DNA constructs of the present invention are members of the family Solanaceae.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis,

-16-

may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term  
5 "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available  
10 for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root  
15 meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The examples which follow are provided to illustrate various specific embodiments of the present invention, and are not to be construed as limiting the  
20 invention.

#### EXAMPLE 1

##### Isolation of Genomic Root Cortex Specific RD2 Genes

A tobacco (*Nicotiana tabacum*) genomic library of DNA isolated from tobacco seedlings was constructed in  
25 EMBL 3 SP6/T7 lambda vector (ClonTech, Palo Alto, CA). TobRD2 cDNA (Conkling et al., *Plant Phys.* 93, 1203 (1990)) was used as a probe to isolate genomic clones containing Tobacco RD2 genes from the primary library. A total of  $1.2 \times 10^7$  recombinant phage were screened on  
30 K802 bacterial cells. The plaques were lifted onto nylon membranes (MagnaGraph), and the DNA immobilized by autoclaving (10 minutes, gravity cycle). All hybridizations were performed at 65°C in aqueous solution (5X SSC [750 mM sodium chloride, 75 mM sodium citrate],  
35 5X Denhardt's [0.1% each of ficoll, BSA, polyvinylpyrrolidone], 0.5% SDS, 100 mg/ml denatured

-17-

salmon sperm DNA) for 16 hours. The filters were washed in 0.2X SSC and 0.1% SDS at 60°C.

Thirteen genomic clones that hybridized to the TobRD2 cDNA probe were identified by screening  $1.2 \times 10^7$  recombinant phage. These clones were isolated and further characterized by restriction mapping. Restriction maps were constructed by the rapid mapping procedure of Rachwitz et al., *Gene*, 30:195 (1984). One clone, homologous to the TobRD2 cDNA, was sequenced in its entirety and its promoter identified. By aligning the TobRD2 cDNA and the genomic clone, the region of the genomic clone 5' to the translated region was identified. The sequence of this untranslated region was examined and the TATAA box of the putative promoter was identified. In plant promoters, the TATAA box is typically -35 to -29 nucleotides from the initiation point of transcription. Using primer extension experiments, the 5' end of transcription was identified.

A 2010 base pair region upstream from the transcribed region of the TobRD2 cDNA is provided in **Figure 2** (SEQ ID NO:1). This sequence includes the predicted start of the transcription region (at nucleotide 2000), and the TATAA box of the promoter (nucleotides 1971-1975).

25

## EXAMPLE 2

### Nucleic Acid Sequencing

Restriction fragments from the isolated genomic clones (Example 1) were subcloned into bluescript (pBS KS II + or pBS SK II+; Stratagene, La Jolla, CA) vectors. Unidirectional deletion series was obtained for each clone and for both DNA strands by Exonuclease III and S1 nuclease digestion (Henikoff, *Gene* 28, 351 (1984). The DNA sequence was determined by dideoxy chain-termination method (Sanger et al., *Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)) using the enzyme Sequenase (U.S.

-18-

Biochemicals, Cleveland, OH). In all cases, both DNA strands were sequenced.

### EXAMPLE 3

#### In-Situ Hybridizations

5 To determine the spatial distribution of TobRD2 mRNA transcripts in the various tissues of the root, *in situ* hybridizations were performed in untransformed plants. In-situ hybridizations of antisense strand of TobRD2 to the TobRD2 mRNA in root tissue was done using  
10 techniques as described in Meyerowitz, *Plant Mol. Biol. Rep.* 5, 242 (1987) and Smith et al., *Plant Mol. Biol. Rep.* 5, 237 (1987). Seven day old tobacco (*Nicotiana tabacum*) seedling roots were fixed in phosphate-buffered glutaraldehyde, embedded in Paraplast Plus (Monoject  
15 Inc., St. Louis, MO) and sectioned at 8 mm thickness to obtain transverse as well as longitudinal sections. Antisense TobRD2 transcripts, synthesized *in vitro* in the presence of 35S-ATP, were used as probes. The labeled RNA was hydrolyzed by alkaline treatment to yield 100 to  
20 200 base mass average length prior to use.

Hybridizations were done in 50% formamide for 16 hours at 42°C, with approximately  $5 \times 10^6$  counts-per-minute (cpm) labeled RNA per milliliter of hybridization solution. After exposure, the slides were developed and  
25 visualized under bright and dark field microscopy.

As shown in Figures 1A and 1B, the hybridization signal is localized to the cortical layer of cells in the roots. Comparison of both bright and dark field images of the same sections localizes TobRD2  
30 transcripts to the parenchymatous cells of the root cortex. No hybridization signal was visible in the epidermis or the stele.

-19-

**EXAMPLE 4****Chimeric Gene Construction**

A promoter deletion series was constructed by polymerase chain reaction (PCR). The templates were the  
5 various deletions of the 5' flanking regions of the TobRD2 genomic clone that had been generated by Exonuclease III/S1 nuclease digestions (Example 2).

All templates were amplified using the same set of oligonucleotide primers. One primer was a modified  
10 bacteriophage M13 forward primer (see, e.g., Sanger et al., *Proc. Natl. Acad. Sci. USA*, 74, 5463 (1977)); the 5' end of the oligonucleotide contained the HindIII recognition sequence, along with an additional 5' sequence that allows for more efficient cleavage by the  
15 restriction enzyme. The other primer was designed to have a BamHI site (along with additional nucleotides for efficient cleavage) at its 5' end and was homologous to the 16 nucleotide sequence of the TobRD2 that is found 22 bases 5' to the ATG start codon (i.e., the primer was  
20 homologous bases 1973-1988 of SEQ ID NO:1).

The PCR amplification reaction contained template plasmid DNA (5-10 ng); reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 [at 25°C], 0.1% Triton X-100, 1.5 mM MgCl); 0.25 mM each of dATP, dGTP, dTTP, and dCTP;  
25 40 ng of each primer; 1.25 units of Taq DNA polymerase (Promega, Madison, WS).

The PCR cycle denatured the templates at 94°C for 1 minute, annealed the primers at 46°C for 1 minute and allowed chain elongation to proceed at 72°C for 5  
30 minutes. This cycle was repeated 40 times and the last elongation cycle was extended by 10 minutes. PCR amplifications were done in a programmable thermal cycler (PTC-100, M.J. Research).

Amplified products were digested with Hind III  
35 and Bam HI and cloned into the Hind III and Bam HI sites of the *Agrobacterium* binary vector pBI 101.3 (R. Jefferson et al., *EMBO J.* 6, 3901-3907 (1987)). This

-20-

vector contains a  $\beta$ -glucuronidase (GUS) reporter gene and an *nptII* selectable marker flanked by the T-DNA border sequences.

#### EXAMPLE 5

##### 5           Plant Transformation: Methods

Chimeric reporter gene constructs were introduced into an *Agrobacterium* host carrying a disarmed Ti-plasmid (LBA4404) capable of providing (*in trans*) the *vir* functions required for T-DNA transfer and integration  
10 into the plant genome, essentially as described by An et al., in S. Belvin and R. Schilperoot, eds., Plant Molecular Biology Manual, Martinus Nijhoff, Dordrecht, The Netherlands, pp A3-1-19 (1988). Constructs were introduced to the host via tri-parental mating or  
15 electroporation of electrocompetant *Agrobacterium* cells, as is known to those in the art. Leaf disc transformation of tobacco (SR1) and plant regeneration were performed as described by An et al. *Plant Physiol.* 81, 301-305 (1986). Kanamycin resistant plants were  
20 selected for further analysis.

#### EXAMPLE 6

##### GUS Assays in Transgenic Plants: Methods

Histochemical staining was performed on excised roots, stems and leaves of transformed plants. The  
25 explant tissues were incubated in 1mM 5-bromo-4-chloro-3-indolyl- B-D-glucuronide (X-Gluc), 25 mM sodium phosphate buffer (pH 7.0), 0.5% DMSO, at 37°C overnight after briefly vacuum infiltrating the substrate. Tissues expressing GUS activity cleave this  
30 substrate and thereby stain blue.

Fluorometric GUS assays were performed as described by Jefferson et al., *EMBO J.* 6, 3901-3907 (1987) to quantitate the level of GUS expression. Cell  
35 extracts from roots, leaves and stems were incubated in the presence of 1 mM 4-methylumbelliferyl-B-D-glucuronide

-21-

(MUG) at 37°C. Samples were taken at 0, 5, 10, 15, and 20 minute intervals. The enzyme reaction was stopped by the addition of 0.2 M sodium carbonate. The fluorometer was calibrated with 10 nM and 100 nM MUG. Protein concentration in the samples was determined according the method of Bradford, *Anal. Biochem.* 72, 248 (1976).

#### EXAMPLE 7

##### Chimeric gene construct is capable of directing tissue-specific gene expression

10 To determine if the 2010 base pair sequence from the TobRD2 gene (SEQ. ID NO:1) encompassed promoter elements directing expression specifically in the parenchymatous cells of the root cortex, chimeric genes were constructed. A 1988 base pair region (SEQ ID NO:2)  
15 was amplified by polymerase chain reaction and cloned 5' to the GUS reporter gene (as described above). The chimeric gene was introduced into tobacco (as described above) and transgenic plants were analyzed for their ability to express GUS (as described above).

20 Results of the analysis of 9 individual transformants (i.e., each transformant was the product of an independent transforming event) are shown in Table 1, lines 25-33 (transformants 325III1 - 325IV5). The  $\Delta$ 2.0 promoter (SEQ ID NO:2) was found to direct high levels of  
25 gene expression (approximately 4-fold higher than that of the CaMV35S promoter, commonly termed to be a 'strong' promoter) (Figure 4). Expression of the reporter could not be detected in leaves or stems at levels higher than control (see Figures 4, 5A and 5B, which display average  
30 activities taken from Table 1). GUS activity was essentially limited to the root and, as shown in Figure 6, was specifically limited to the root cortex. The plant shown in Figure 6 was transformed using the  $\Delta$ 2.0 promoter driving GUS, in pBI101.3.

35 (Multiple individual transformed leaf disks were placed in petri plates. Transformant nomenclature



-22-

in **Table 1** indicates the promoter/the numbered petri plate/and the number of the independent transformant. Thus 325II1 refers to a transformant using the  $\Delta$ 2.0 promoter, in petri plate II, and from leaf disc 1; while

5 101.I1 refers to transformation using pBI101.3 (promoterless GUS used as a control), and to transformant number 1 in petri plate I. In **Table 1**, the prefix 121 refers to use of pBI121 (CaMV35S promoter with GUS); 325 refers to the  $\Delta$ 2.0 promoter (SEQ ID NO:2) with GUS; 484

10 refers to the  $\Delta$ 1.4 promoter (SEQ ID NO:3) with GUS; 421 refers to the  $\Delta$ 1.3 promoter (SEQ ID NO:4) with GUS; 428 refers to the  $\Delta$ 1.0 promoter (SEQ ID NO:5) with GUS; 490 refers to the  $\Delta$ 0.7 promoter (SEQ ID NO:6) with GUS; 491 refers to the  $\Delta$ 0.6 promoter (SEQ ID NO:7) with GUS; 492

15 refers to the  $\Delta$ 0.5 promoter (SEQ ID NO:8) with GUS; 495 refers to the  $\Delta$ 0.2 promoter (SEQ ID NO:9) with GUS. "R-GUS" refers to GUS activity in root tissues; "L-GUS" refers to GUS activity in leaf tissues; and "S-GUS" refers to GUS activity in stem tissues. R/L provides the

20 relative GUS activity in Roots/Leaves; R/S provides the relative GUS activity in Roots/Stems. GUS activity is provided in pmolMU/ $\mu$ g protein/min.

- 23 -

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

Transformants	R-GUS activity	Average	L-GUS activity	Average	S-GUS activity	Average	R/L	R/L mean	R/S	R/S mean
101.II	0.19	0.56	0.23	0.33	0.22	0.36	0.83	1.67	0.86	1.51
101.I2	0.12		0.14		0.15		0.66		0.80	
101.I3	0.13		0.35		0.32		0.37		0.41	
101.I4	0.73		0.46		0.24		1.59		3.04	
101.II1	0.44				0.31				1.42	
101.II3	0.59		0.23		0.47		2.57		1.26	
101.II4	0.86		0.41		0.34		2.10		2.53	
101.II5	0.84		0.36		0.33		1.78		1.94	
101.III1	0.69		0.24		0.42		2.88		1.64	
101.III3	0.25		0.19		0.21		1.32		1.19	
101.III4	0.71		0.37		0.27		1.92		2.63	
101.III5	0.15		0.13		0.21		1.15		0.71	
101.IV1	0.21		0.10		0.13		2.10		1.62	
101.IV2	0.27		0.24		0.23		1.13		1.17	
101.IV3	0.88		0.42		0.57		2.10		1.54	
101.IV4	0.75		0.35		0.67		2.14		1.12	
101.IV5	1.88		0.98		1.02		1.92		1.84	
121.I5	3.00	10.50	3.65	14.36	2.25	5.81	0.82	0.71	1.33	1.69
121.IV1	24.67		30.79		11.96		0.80		2.06	
121.IV2	9.20		11.66		5.33		0.79		1.73	
121.IV4	12.13		15.61		7.42		0.78		1.63	
121.4	3.50		10.10		2.08		0.35		1.68	

-24-

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

325II1	35.30	32.15	0.54	0.46	0.61	0.78	65.37	67.19	57.87	50.17
325II2	24.94		0.24		0.35		103.92		71.26	
325II4	13.64		0.17		0.23		80.24		59.30	
325II5	38.09				0.64				59.52	
325III1	45.31		0.38							
325III2	34.05		0.44							
325III5	55.81		0.76		0.77		73.43		72.48	
325IV1	16.51		0.68		0.94		24.28		17.56	
325IV5	25.71		0.46		1.95		55.89		13.18	
484I1	61.75	36.68		0.46		0.67		74.41		53.68
484I3	59.72									
484I4	72.35									
484I5	56.58									
484V2	38.32		0.78		0.86		49.13		44.56	
484V3	23.68		0.31		2.29		76.32		10.33	
484III3	63.28									
484III4	42.91		0.87		0.98		49.32		43.79	
484II4	15.80		0.43		0.27		36.74		58.52	
484V4	58.25		0.46		0.48		126.63		121.35	
484V1	26.86		0.81		1.27		33.16		21.15	
484V5	8.53		0.42		0.34		20.31		25.09	
484IV5	17.83		0.51		0.29		34.96		61.48	
484IV3	14.05		0.35		0.34		40.14		41.32	
484IV2	32.33		0.32		0.51		101.03		63.39	

- 25 -

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

484II3	10.18		0.13		0.16		78.31		63.63	
484II5	33.51		0.55		0.63		60.93		53.19	
484II2	52.54		0.43		0.79		122.19		66.51	
484II1	8.50		0.04		0.11		212.50		77.27	
421IV4	25.04	31.87	0.82	0.81	2.27	1.01	30.54	40.54	11.03	36.78
421IV4	46.31		0.82				56.48			
421II4	79.23		0.96		1.89		82.53		41.92	
421III3	17.00		0.45		1.09		37.78		15.60	
421II3	19.07		0.42		0.37		45.40		51.54	
421II1	27.87		0.72		0.64		38.43		43.23	
421I3	74.45		2.27		1.44		32.80		51.70	
421I2	43.36		0.88		0.56		49.27		77.43	
421I4	8.41									
421V1	32.32		0.94		1.34		34.38		24.12	
421V2	5.07		0.43		0.13		11.79		39.00	
421IV3	4.52		0.17		0.37		26.59		12.22	
428I5	20.62	38.64	0.98	0.66	0.83	0.65	21.04	72.65	24.84	47.43
428I2	15.05		0.97		0.25		15.52		60.20	
428III3	69.87		1.10				63.52			
428III1	30.97		0.52		0.36		59.56		86.03	
428V2	54.66		0.24				227.75			
428V1	85.71		0.98		1.25		87.46		68.57	
428IV4	4.15				0.29				14.31	

-26-

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

428IV5	26.42		0.43			1.10		61.44		24.02	
428V3	1.68		0.18			0.17		9.88		9.29	
428V2	25.60		0.34					75.29			
428III5	90.36		0.86			0.98		105.07		92.20	
490II4	9.38	22.77		0.54			0.75		41.65		38.11
490II5	9.87		0.35			0.85		27.63		14.88	
490I1	33.62		0.93			2.02		38.15		16.64	
490I2	34.68		0.98			1.13		35.37		30.67	
490I3	4.58										
490III2	76.74										
490III4	58.75		1.07			1.21		54.91		48.55	
490III5	6.65		0.21			0.09		31.67		73.89	
490IV2	12.24										
490I11	8.09		0.22			0.21		36.77		38.52	
490IV4	20.19		0.35			0.52		57.69		38.83	
490IV5	17.57		0.34			0.57		51.68		30.82	
490IV3	18.11										
490I5	23.03		0.78			0.93		29.53		24.76	
490V5	8.27		0.15			0.19		55.13		43.53	
491I2	8.31	39.76		0.50			0.63		53.70		45.85
491II3	6.73										
491II4	13.01		0.23			0.19		56.57		68.47	
491V5	87.40										

-27-

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

491IV1	77.12		1.02			1.34		75.61		57.55	
491IV3	49.20		0.98			1.23		50.20		40.00	
491III1	18.84		0.32			0.34		58.88		55.41	
491III2	30.82		0.47			0.58		65.57		53.14	
491III5	8.46		0.28			.045		30.21		18.80	
491IV5	2.88										
491III5	8.55		0.22			0.31		28.86		27.58	
491IV4	165.77										
492V2	2.40	9.89	0.21	0.57		0.24	0.54	11.43	15.59	10.00	16.72
492V4	3.17		0.27			0.48		11.74		6.60	
492I3	4.40		0.87			0.35		5.06		12.57	
492I4	6.58		0.50			0.37		13.16		17.78	
492I5	10.26										
492III2	11.87		0.78			1.06		15.22		11.20	
492IV4	7.38										
492IV5	21.63										
492III5	11.39		0.61			0.32		18.67		35.59	
492IV1	20.38		0.81			0.94		25.16		21.68	
492I3	12.15		0.42			0.53		28.93		22.92	
492III1	7.03		0.64			0.58		10.98		12.12	
495I1	3.58	5.83	0.37	0.41		0.43	0.54	9.68	17.98	8.33	13.35
495I3	16.41		0.59			0.74		27.81		22.18	
495I4	3.20		0.17			0.17		18.82		18.82	

TABLE 1  
TOBRD2 PROMOTER ANALYSIS

495I5	5.98	0.32		0.34	18.63	17.53	
495II2	8.49	0.54		0.52	15.72	16.33	
495III2	5.12	0.40		0.77	12.80	6.65	
495IV1	5.57	0.21		0.45	26.52	12.38	
495IV2	9.74	0.75		1.03	12.99	9.46	
495IV3	2.64	0.14		0.31	18.66	8.52	
495IV4	1.20						
495V1	3.67						
495V2	2.38						
495V3	7.60						
495V4	6.10	0.56		0.62	10.89	9.84	

-29-

**EXAMPLE 8****Effect of 5' promoter-deletions  
on the expression of the reporter gene activity**

The following experiments were carried out in essentially the same manner as described in Example 7, above, except that the length of the TobRD2 flanking region employed as a promoter was varied to explore how various portions of the flanking region affected expression of GUS

A series of seven nested 5'-deletion mutations in the 2010 base pair TobRD2 sequence (SEQ ID NO:1) upstream region were generated for use as promoter sequences. These deletion mutants are shown graphically in Figure 3, and are denoted as  $\Delta 2.0$  (SEQ ID NO:2);  $\Delta 1.4$  (SEQ ID NO:3);  $\Delta 1.3$  (SEQ ID NO:4);  $\Delta 1.0$  (SEQ ID NO:5);  $\Delta 0.7$  (SEQ ID NO:6);  $\Delta 0.6$  (SEQ ID NO:7);  $\Delta 0.5$  (SEQ ID NO:8); and  $\Delta 0.2$  (SEQ ID NO:9).

Chimeric gene constructs as described in Example 3 and containing the  $\Delta 2.00$  promoter (SEQ ID NO:2) or a truncated promoter (SEQ ID NOs: 3-9) were introduced into tobacco by *Agrobacterium* mediated transformation of leaf discs (as described in Example 4). The *Agrobacterium* vector pBI101.3 was used alone as a control, and the CaMV35S promoter was used to provide a reference standard. Roots, leaves and stems from regenerated plants were assayed for GUS activity (Table 1; Fig. 4).

Figure 4 provides a graphic representation of GUS activity in roots, leaves and stems using the full length TobRD2 promoter, the promoter deletion series, the Cauliflower Mosaic Virus 35S (CaMV35S) promoter, and vector pBI101.3 as a control. As shown in Figure 4, six of the promoters tested were found to confer high levels of root cortex specific expression:  $\Delta 2.00$  (SEQ ID NO:2);  $\Delta 1.4$  (SEQ ID NO:3);  $\Delta 1.3$  (SEQ ID NO:4);  $\Delta 1.0$  (SEQ ID NO:5);  $\Delta 0.7$  (SEQ ID NO:6); and  $\Delta 0.6$  (SEQ ID NO:7). Figure 4 displays averaged data from Table 1.



-30-

As further shown in **Figure 4**, loss of a region approximately 50 base pairs in length (compare  $\Delta 0.6$  (SEQ ID NO:7) and  $\Delta 0.5$  (SEQ ID NO:8)) drastically decreased the level of GUS expression. However, the results show  
5 that the level of GUS expression in root tissue provided by the  $\Delta 0.5$  promoter (SEQ ID NO:8) was equivalent to that elicited by the CaMV35S promoter. GUS expression in root cortex provided by the  $\Delta 0.2$  promoter (SEQ ID NO:9) was approximately half that provided by the CaMV35S promoter.

10 **Figures 5A and 5B** further illustrate the organ specific nature of reporter gene expression using TobRD2 promoters. In all instances tested, GUS activity was strictly expressed in the roots and negligible activity, if any, was detected in the stems or leaves of the same  
15 transformed tobacco plants. While the level of GUS activity measured in roots transformed with the  $\Delta 0.60$  and  $\Delta 0.30$  promoters was equivalent to or less than that provided by the CaMV35S promoter (**Figure 4**), **Figures 5A and 5B** illustrate that expression directed by the  $\Delta 0.60$   
20 and  $\Delta 0.30$  promoters was root-specific, with negligible activity in stems and leaves, unlike expression directed by the CaMV35S promoter.

The foregoing examples are illustrative of the present invention, and are not to be construed as  
25 limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

-31-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Conkling, Mark A.  
Mendu, Nandini  
Song, Wen
- (ii) TITLE OF INVENTION: Root Cortex Specific Gene Promoter
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park & Gibson
  - (B) STREET: Post Office Drawer 34009
  - (C) CITY: Charlotte
  - (D) STATE: North Carolina
  - (E) COUNTRY: USA
  - (F) ZIP: 28234
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0. Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Sibley, Kenneth D.
  - (B) REGISTRATION NUMBER: 31,665
  - (C) REFERENCE/DOCKET NUMBER: 5051-294
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 919-420-2200
  - (B) TELEFAX: 919-881-3175

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2010 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

- 32 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGAGGATC TAAATTGTGA GTTCAATCTC TTCCCTATTG GATTGATTAT CCTTTCTTTT	60
CTTCCAATTT GTGTTTCTTT TTGCCTAATT TATTGTGTTA TCCCCTTTAT CCTATTTTGT	120
TTCTTTACTT ATTTATTTGC TTCTATGTCT TTGTACAAAG ATTTAAACTC TATGGCACAT	180
ATTTTAAAGT TGTTAGAAAA TAAATTCTTT CAAGATTGAT GAAAGAACTT TTTAATTGTA	240
GATATTTTCGT AGATTTTATT CTCTTACTAC CAATATAACG CTTGAATTGA CGAAAATTTG	300
TGTCCAAATA TCTAGCAAAA AGGTATCCAA TGAAAATATA TCATATGTGA TCTTCAAATC	360
TTGTGTCTTA TGCAAGATTG ATACTTTGTT CAATGGAAGA GATTGTGTGC ATATTTTAA	420
AATTTTATT AGTAATAAAG ATTCTATATA GCTGTTATAG AGGGATAATT TTACAAAGAA	480
CACTATAAAT ATGATTGTTG TTGTTAGGGT GTCAATGGTT CGGTTGACT GGTATTTTA	540
TAAAATTTGT ACCATACCAT TTTTTCGAT ATTCTATTTT GTATAACCAA AATTAGACTT	600
TTCGAAATCG TCCAATCAT GTCGGTTTCA CTTCGGTATC GGTACCGTTC GGTTAATTTT	660
CATTTTTTTT TAAATGTCAT TAAATTCAC TAGTAAAAAT AGAATGCAAT AACATACGTT	720
CTTTTATAGG ACTTAGCAAA AGCTCTCTAG ACATTTTAC TGTTTAAAGG ATAATGAATT	780
AAAAACATG AAAGATGGCT AGAGTATAGA TACACAATA TTCGACAGCA ACGTAAAAGA	840
AACCAAGTAA AAGCAAAGAA AATATAAATC ACACGAGTGG AAAGATATTA ACCAAGTTGG	900
GATTCAAGAA TAAAGTCTAT ATTAAATATT CAAAAAGATA AATTAAATA ATATGAAAGG	960
AAACATATTC AATACATTGT AGTTTGCTAC TCATAATCGC TAGAATACTT TGTGCCTTGC	1020
TAATAAGAT ACTTGAAATA GCTTAGTTTA AATATAAATA GCATAATAGA TTTTAGGAAT	1080
TAGTATTTTG AGTTTAATTA CTTATTGACT TGTAACAGTT TTTATAATTC CAAGGCCCAT	1140
GAAAAATTTA ATGCTTTATT AGTTTAAAC TTAATATATA AATTTTTCAT ATGTAAAATT	1200
TAATCGGTAT AGTTCGATAT TTTTCAATT TATTTTATA AAATAAAAAA CTTACCCTAA	1260
TTATCGGTAC AGTTATAGAT TTATATAAAA ATCTACGGTT CTTCAGAAGA AACCTAAAAA	1320
TCGGTTCGGT GCGGACGGTT CGATCGGTTT AGTCGATTTT CAAATATTCA TTGACACTCC	1380
TAGTTGTTGT TATAGGTAAA AAGCAGTTAC AGAGAGGTAA AATATAACTT AAAAAATCAG	1440
TTCTAAGGAA AAATTGACTT TTATAGTAAA TGAAGTTAT ATAAGGATGT TGTTACAGAG	1500
AGGTATGAGT GTAGTTGGTA AATTATGTTT TTAGCGGTGT ATGTCACATA TTATTTATTA	1560
AAACTAGAAA AAACAGCGTC AAAACTAGCA AAAATCCAAC GGACAAAAAA ATCGGCTGAA	1620

- 33 -

TTTGATTTGG TTCCAACATT TAAAAAGTT TCAGTGAGAA AGAATCGGTG ACTGTTGATG	1680
ATATAAACAA AGGGCACATT GGTCATAAC CATAAAAAAT TATATGACAG CTACAGTTGG	1740
TAGCATGTGC TCAGCTATTG AACAAATCTA AAGAAGGTAC ATCTGTAACC GGAACACCAC	1800
TTAAATGACT AAATTACCCT CATCAGAAAG CAGATGGAGT GCTACAAATA ACACACTATT	1860
CAACAACCAT AAATAAACG TGTTCACTA CTAACAAAA TATAAATAA TCTATGTTTG	1920
TAAGCACTCC AGCCATGTTA ATGGAGTGCT ATTGCCTGTT AACTCTCACT TATAAATAG	1980
TAGTAGAAAA AATATGAACC AAAACACAAC	2010

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1988 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCGAGGATC TAAATTGTGA GTTCAATCTC TTCCCTATTG GATTGATTAT CCTTTCTTTT	60
CTTCCAATTT GTGTTTCTTT TTGCCTAATT TATTGTGTTA TCCCCTTTAT CCTATTTTGT	120
TTCTTTACTT ATTTATTTGC TTCTATGTCT TTGTACAAAG ATTTAACTC TATGGCACAT	180
ATTTTAAAGT TGTTAGAAAA TAAATTCTTT CAAGATTGAT GAAAGAACTT TTTAATTGTA	240
GATATTTTCT AGATTTTATT CTCTTACTAC CAATATAACG CTTGAATTGA CGAAAATTTG	300
TGTCCAAATA TCTAGCAAAA AGGTATCCAA TGAAAATATA TCATATGTGA TCTTCAAATC	360
TTGTGTCTTA TGCAAGATTG ATACTTTGTT CAATGGAAGA GATTGTGTGC ATATTTTAA	420
AATTTTATT AGTAATAAAG ATTCTATATA GCTGTTATAG AGGGATAATT TTACAAAGAA	480
CACTATAAAT ATGATTGTTG TTGTTAGGGT GTCAATGGTT CGGTTCGACT GGTTATTTTA	540
TAAAATTTGT ACCATACCAT TTTTTCGAT ATTCTATTTT GTATAACCAA AATTAGACTT	600
TTCGAAATCG TCCAATCAT GTCGGTTTCA CTTCGGTATC GGTACCGTTC GGTTAATTTT	660
CATTTTTTTT TAAATGTCAT TAAATTCAC TAGTAAAAAT AGAATGCAAT AACATACGTT	720
CTTTTATAGG ACTTAGCAAA AGCTCTCTAG ACATTTTAC TGTTTAAAGG ATAATGAATT	780

- 34 -

AAAAACATG AAAGATGGCT AGAGTATAGA TACACAATA TCGACAGCA ACGTAAAAGA	840
AACCAAGTAA AAGCAAAGAA AATATAAATC ACACGAGTGG AAAGATATTA ACCAAGTTGG	900
GATTCAAGAA TAAAGTCTAT ATTAAATATT CAAAAAGATA AATTTAAATA ATATGAAAGG	960
AAACATATTC AATACATTGT AGTTTGCTAC TCATAATCGC TAGAATACTT TGTGCCTTGC	1020
TAATAAGAT ACTTGAAATA GCTTAGTTTA AATATAAATA GCATAATAGA TTTTAGGAAT	1080
TAGTATTTTG AGTTTAATTA CTTATTGACT TGTAACAGTT TTTATAATTC CAAGGCCCAT	1140
GAAAAATTTA ATGCTTTATT AGTTTAAAC TTAATATATA AATTTTTCAT ATGTAAAATT	1200
TAATCGGTAT AGTTCGATAT TTTTCAATT TATTTTATA AAATAAAAAA CTTACCCTAA	1260
TTATCGGTAC AGTTATAGAT TTATATAAAA ATCTACGGTT CTTGAGAAGA AACCTAAAAA	1320
TCGGTTCGGT GCGGACGGTT CGATCGGTTT AGTCGATTTT CAAATATTCA TTGACACTCC	1380
TAGTTGTTGT TATAGGTAAA AAGCAGTTAC AGAGAGGTAA AATATAACTT AAAAAATCAG	1440
TTCTAAGGAA AAATTGACTT TTATAGTAAA TGACTGTTAT ATAAGGATGT TGTTACAGAG	1500
AGGTATGAGT GTAGTTGGTA AATTATGTTT TTGACGGTGT ATGTCACATA TTATTTATTA	1560
AACTAGAAA AAACAGCGTC AAACTAGCA AAAATCCAAC GGACAAAAAA ATCGGCTGAA	1620
TTTGATTTGG TTCCAACATT TAAAAAGTT TCAGTGAGAA AGAATCGGTG ACTGTTGATG	1680
ATATAACAA AGGGCACATT GGTCAATAAC CATAAAAAAT TATATGACAG CTACAGTTGG	1740
TAGCATGTGC TCAGCTATTG AACAAATCTA AAGAAGGTAC ATCTGTAACC GGAACACCAC	1800
TTAAATGACT AAATTACCCT CATCAGAAAG CAGATGGAGT GCTACAAATA ACACACTATT	1860
CAACAACCAT AAATAAACG TGTTCAAGCTA CTAACAACAA TATAAATAAA TCTATGTTTG	1920
TAAGCACTCC AGCCATGTTA ATGGAGTGCT ATTGCCTGTT AACTCTCACT TATAAATAG	1980
TAGTAGAA	1988

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1372 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

-35-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCATGTCGGT TTCACTTCGG TATCGGTACC GTTCGGTTAA TTTTCATTTT TTTTAAATG	60
TCATTAATAAT TCACTAGTAA AAATAGAATG CAATAACATA CGTTCTTTTA TAGGACTTAG	120
CAAAAGCTCT CTAGACATTT TTAAGTGTAA AAGGATAATG AATTAAAAAA CATGAAAGAT	180
GGCTAGAGTA TAGATACACA ACTATTCGAC AGCAACGTAA AAGAAACCAA GTAAAAGCAA	240
AGAAAATATA AATCACACGA GTGGAAAGAT ATTAACCAAG TTGGGATTCA AGAATAAAGT	300
CTATATTAAT TATTCAAAAA GATAAATTTA AATAATATGA AAGGAAACAT ATTCAATACA	360
TTGTAGTTTG CTACTCATAA TCGCTAGAAT ACTTTGTGCC TTGCTAATAA AGATACTTGA	420
AATAGCTTAG TTTAAATATA AATAGCATAA TAGATTTTAG GAATTAGTAT TTTGAGTTTA	480
ATTACTTATT GACTTGTAAC AGTTTTTATA ATTCCAAGGC CCATGAAAAA TTTAATGCTT	540
TATTAGTTTT AAACCTACTA TATAAATTTT TCATATGTAA AATTTAATCG GTATAGTTTCG	600
ATATTTTTTC AATTTATTTT TATAAAATAA AAAACTTACC CTAATTATCG GTACAGTTAT	660
AGATTTATAT AAAAATCTAC GGTTCCTCAG AAGAAACCTA AAAATCGGTT CGGTGCGGAC	720
GGTTCGATCG GTTTAGTCGA TTTTCAAATA TTCATTGACA CTCCTAGTTG TTGTTATAGG	780
TAAAAAGCAG TTACAGAGAG GTAAAATATA ACTTAAAAAA TCAGTTCTAA GGAAAAATTG	840
ACTTTTATAG TAAATGACTG TTATATAAGG ATGTTGTTAC AGAGAGGTAT GAGTGTAGTT	900
GGTAAATTAT GTTCTTGACG GTGTATGTCA CATATTATTT ATTAAACTA GAAAAACAG	960
CGTCAAAACT AGCAAAAATC CAACGGACAA AAAAATCGGC TGAATTTGAT TTGGTTCCAA	1020
CATTTAAAAA AGTTTCAGTG AGAAAGAATC GGTGACTGTT GATGATATAA ACAAAGGGCA	1080
CATTGGTCAA TAACCATAAA AAATTATATG ACAGCTACAG TTGGTAGCAT GTGCTCAGCT	1140
ATTGAACAAA TCTAAAGAAG GTACATCTGT AACCGGAACA CCACTTAAAT GACTAAATTA	1200
CCCTCATCAG AAAGCAGATG GAGTGCTACA AATAACACAC TATTCAACAA CCATAAATAA	1260
AACGTGTTCA GCTACTAAAA CAAATATAAA TAAATCTATG TTTGTAAGCA CTCCAGCCAT	1320
GTTAATGGAG TGCTATTGCC TGTTAACTCT CACTTATAAA ATAGTAGTAG AA	1372

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1294 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

-36-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAAAATAGAA TGCAATAACA TACGTTCTTT TATAGGACTT AGCAAAAGCT CTCTAGACAT	60
TTTACTGTT TAAAGGATAA TGAATTA AAA AACATGAAAG ATGGCTAGAG TATAGATACA	120
CAACTATTCG ACAGCAACGT AAAAGAAACC AAGTAAAGC AAAGAAAATA TAAATCACAC	180
GAGTGGAAG ATATTAACCA AGTTGGGATT CAAGAATAAA GTCTATATTA AATATTCAAA	240
AAGATAAATT TAAATAATAT GAAAGGAAAC ATATTCAATA CATTGTAGTT TGCTACTCAT	300
AATCGCTAGA ATACTTTGTG CCTTGCTAAT AAAGATACTT GAAATAGCTT AGTTTAAATA	360
TAAATAGCAT AATAGATTTT AGGAATTAGT ATTTTGAGTT TAATTACTTA TTGACTTGTA	420
ACAGTTTTTA TAATTCCAAG GCCCATGAAA AATTTAATGC TTTATTAGTT TTAAACTTAC	480
TATATAAATT TTTCATATGT AAAATTTAAT CGGTATAGTT CGATATTTTT TCAATTTATT	540
TTTATAAAT AAAAACTTA CCCTAATTAT CGGTACAGTT ATAGATTTAT ATAAAAATCT	600
ACGGTTCCTC AGAAGAAACC TAAAAATCGG TTCGGTGCGG ACGGTCGAT CGGTTTAGTC	660
GATTTTCAA TATTCATTGA CACTCCTAGT TGTGTGTATA GGTAAAAAGC AGTTACAGAG	720
AGGTAAAATA TAACTTAAAA AATCAGTTCT AAGGAAAAAT TGACTTTTAT AGTAAATGAC	780
TGTTATATAA GGATGTTGTT ACAGAGAGGT ATGAGTGTAG TTGGTAAATT ATGTTCTTGA	840
CGGTGTATGT CACATATTAT TTATTA AAAC TAGAAAAAAC AGCGTCAAAA CTAGCAAAAA	900
TCCAACGGAC AAAAAATCG GCTGAATTTG ATTTGGTTCC AACATTTAAA AAAGTTTCAG	960
TGAGAAAGAA TCGGTGACTG TTGATGATAT AAACAAAGGG CACATTGGTC AATAACCATA	1020
AAAAATTATA TGACAGCTAC AGTTGGTAGC ATGTGCTCAG CTATTGAACA AATCTAAAGA	1080
AGGTACATCT GTAACCGGAA CACCACTTAA ATGACTAAAT TACCCTCATC AGAAAGCAGA	1140
TGGAGTGCTA CAAATAACAC ACTATTCAAC AACCATAAAT AAAACGTGTT CAGCTACTAA	1200
AACAAATATA AATAAATCTA TGTTTGTAAG CACTCCAGCC ATGTTAATGG AGTGCTATTG	1260
CCTGTAACT CTCATTATA AAATAGTAGT AGAA	1294

-37-

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1030 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGAAACATAT TCAATACATT GTAGTTTGCT ACTCATAATC GCTAGAATAC TTTGTGCCTT	60
GCTAATAAAG ATACTTGAAA TAGCTTAGTT TAAATATAAA TAGCATAATA GATTTTAGGA	120
ATTAGTATTT TGAGTTTAAT TACTTATTGA CTTGTAACAG TTTTATAAT TCCAAGGCC	180
ATGAAAAATT TAATGCTTTA TTAGTTTTAA ACTTACTATA TAAATTTTTC ATATGTAAAA	240
TTTAATCGGT ATAGTTCGAT ATTTTTTCAA TTTATTTTTA TAAAATAAAA AACTTACCCT	300
AATTATCGGT ACAGTTATAG ATTTATATAA AAATCTACGG TTCTTCAGAA GAAACCTAAA	360
AATCGGTTTC GTGCGGACGG TTCGATCGGT TTAGTCGATT TTCAAATATT CATTGACACT	420
CCTAGTTGTT GTTATAGGTA AAAAGCAGTT ACAGAGAGGT AAAATATAAC TTAATAAATC	480
AGTTCTAAGG AAAAATTGAC TTTTATAGTA AATGACTGTT ATATAAGGAT GTTGTTACAG	540
AGAGGTATGA GTGTAGTTGG TAAATTATGT TCTTGACGGT GTATGTCACA TATTATTTAT	600
TAAACTAGA AAAACAGCG TCAAACTAG CAAAATCCA ACGGACAAAA AAATCGGCTG	660
AATTTGATTT GGTTCCAACA TTTAAAAAAG TTTCAGTGAG AAAGAATCGG TGA CTGTTGA	720
TGATATAAAC AAAGGGCACA TTGGTCAATA ACCATAAAAA ATTATATGAC AGCTACAGTT	780
GGTAGCATGT GCTCAGCTAT TGAACAAATC TAAAGAAGGT ACATCTGTAA CCGGAACACC	840
ACTTAAATGA CTAAATTACC CTCATCAGAA AGCAGATGGA GTGCTACAAA TAACACACTA	900
TTCAACAACC ATAAATAAAA CGTGTTTCAGC TACTAAAACA AATATAAATA AATCTATGTT	960
TGTAAGCACT CCAGCCATGT TAATGGAGTG CTATTGCCTG TTA ACTCTCA CTTATAAAAT	1020
AGTAGTAGAA	1030



-38-

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 722 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTACAGTTAT AGATTTATAT AAAAATCTAC GGTTCCTCAG AAGAAACCTA AAAATCGGTT	60
CGGTGCGGAC GGTTCGATCG GTTTAGTCGA TTTTCAAATA TTCATTGACA CTCCTAGTTG	120
TTGTTATAGG TAAAAAGCAG TTACAGAGAG GTAAAATATA ACTTAAAAAA TCAGTTCTAA	180
GGAAAAATTG ACTTTTATAG TAAATGACTG TTATATAAGG ATGTTGTTAC AGAGAGGTAT	240
GAGTGTAGTT GGTAAATTAT GTTCTTGACG GTGTATGTCA CATATTATTT ATTAAAACTA	300
GAAAAACAG CGTCAAAACT AGCAAAAATC CAACGGACAA AAAAATCGGC TGAATTTGAT	360
TTGGTTCCAA CATTTAAAAA AGTTTCAGTG AGAAAGAATC GGTGACTGTT GATGATATAA	420
ACAAAGGGCA CATTGGTCAA TAACCATAAA AAATTATATG ACAGCTACAG TTGGTAGCAT	480
GTGCTCAGCT ATTGAACAAA TCTAAAGAAG GTACATCTGT AACCGGAACA CCACTTAAAT	540
GACTAAATTA CCCTCATCAG AAAGCAGATG GAGTGCTACA AATAACACAC TATTCAACAA	600
CCATAAATAA AACGTGTTCA GCTACTAAAA CAAATATAAA TAAATCTATG TTTGTAAGCA	660
CTCCAGCCAT GTTAATGGAG TGCTATTGCC TGTTAACTCT CACTTATAAA ATAGTAGTAG	720
AA	722

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 574 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

-39-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGTAAAATA TAACTTAAAA AATCAGTTCT AAGGAAAAAT TGACTTTTAT AGTAAATGAC	60
TGTTATATAA GGATGTTGTT ACAGAGAGGT ATGAGTGTAG TTGGTAAATT ATGTTCTTGA	120
CGGTGTATGT CACATATTAT TTATTAAAC TAGAAAAAC AGCGTCAAAA CTAGCAAAAA	180
TCCAACGGAC AAAAAATCG GCTGAATTTG ATTTGGTTCC AACATTTAAA AAAGTTTCAG	240
TGAGAAAGAA TCGGTGACTG TTGATGATAT AAACAAAGGG CACATTGGTC AATAACCATA	300
AAAAATTATA TGACAGCTAC AGTTGGTAGC ATGTGCTCAG CTATTGAACA AATCTAAAGA	360
AGGTACATCT GTAACCGGAA CACCACTTAA ATGACTAAAT TACCCTCATC AGAAAGCAGA	420
TGGAGTGCTA CAAATAACAC ACTATTCAAC AACCATAAAT AAAACGTGTT CAGCTACTAA	480
AACAAATATA AATAAATCTA TGTTTGTAAG CACTCCAGCC ATGTTAATGG AGTGCTATTG	540
CCTGTAACT CTCACCTATA AAATAGTAGT AGAA	574

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 523 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTAAATGACT GTTATATAAG GATGTTGTTA CAGAGAGGTA TGAGTGTAGT TGGTAAATTA	60
TGTTCTTGAC GGTGTATGTC ACATATTATT TATTAAAACT AGAAAAAACA GCGTCAAAAC	120
TAGCAAAAAT CCAACGGACA AAAAAATCGG CTGAATTTGA TTTGGTTCCA ACATTTAAAA	180
AAGTTTCAGT GAGAAAGAAT CGGTGACTGT TGATGATATA AACAAAGGGC ACATTGGTCA	240
ATAACCATAA AAAATTATAT GACAGCTACA GTTGGTAGCA TGTGCTCAGC TATTGAACAA	300
ATCTAAAGAA GGTACATCTG TAACCGGAAC ACCACTTAAA TGAATAAATT ACCCTCATCA	360
GAAAGCAGAT GGAGTGCTAC AAATAACACA CTATTCAACA ACCATAAATA AACGTGTTC	420
AGCTACTAAA ACAAATATAA ATAAATCTAT GTTTGTAAGC ACTCCAGCCA TGTTAATGGA	480
GTGCTATTGC CTGTTAACTC TCACTTATAA AATAGTAGTA GAA	523

- 40 -

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 220 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TAAAGAAGGT ACATCTGTAA CCGGAACACC ACTTAAATGA CTAAATTACC CTCATCAGAA	60
AGCAGATGGA GTGCTACAAA TAACACACTA TTCAACAACC ATAAATAAAA CGTG TTCAGC	120
TACTAAAACA AATATAAATA AATCTATGTT TGTAAGCACT CCAGCCATGT TAATGGAGTG	180
CTATTGCCTG TTAACCTCTCA CTTATAAAAT AGTAGTAGAA	220

-41-

## THAT WHICH IS CLAIMED IS:

1. An isolated DNA molecule which directs root cortex specific transcription of a downstream heterologous DNA segment in a plant cell, said isolated DNA molecule having a sequence selected from the group  
5 consisting of:

(a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 provided herein, and

(b) DNA sequences which hybridize to isolated  
10 DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°, and which direct root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

15 2. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a Tobacco RD2 promoter and a heterologous DNA segment positioned downstream from said promoter and operatively associated therewith.

20 3. A DNA construct comprising an expression cassette, which construct comprises in the 5' to 3' direction, a root cortex specific promoter and a heterologous DNA segment positioned downstream from said promoter and operatively associated therewith, wherein  
25 said root cortex specific promoter has a sequence selected from the group consisting of:

(a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 provided herein, and

30 (b) DNA sequences which hybridize to isolated DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°, and which direct root

-42-

cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

4. A DNA construct according to claim 3, wherein said construct further comprises a plasmid.

5 5. A DNA construct according to claim 3, wherein said heterologous DNA segment is a gene coding for an insecticidal protein.

6. A DNA construct according to claim 4, wherein said heterologous DNA segment is a gene coding  
10 for a *Bacillus thuringiensis* crystal protein toxic to insects.

7. A plant cell containing a DNA construct according to claim 3.

8. A method of making a transformed plant,  
15 comprising regenerating a plant from a plant cell according to claim 7.

9. An *Agrobacterium tumefaciens* cell containing a DNA construct according to claim 3, and wherein said DNA construct further comprises a Ti  
20 plasmid.

10. A method of making a transformed plant, comprising infecting a plant cell with an *Agrobacterium tumefaciens* according to claim 9 to produce a transformed plant cell, and then regenerating a plant from said  
25 transformed plant cell.

11. A microparticle carrying a DNA construct according to claim 3, wherein said microparticle is suitable for the ballistic transformation of a plant cell.

-43-

12. A method of making a transformed plant, comprising propelling a microparticle according to claim 11 into a plant cell to produce a transformed plant cell, and then regenerating a plant from said transformed plant  
5 cell.

13. A plant cell protoplast containing a DNA construct according to claim 3.

14. A method of making a transformed plant, comprising regenerating a plant from a plant cell  
10 protoplast according to claim 13.

15. A transformed plant comprising transformed plant cells, said transformed plant cells containing a heterologous DNA construct, which construct comprises in the 5' to 3' direction, a root cortex specific promoter  
15 and a heterologous DNA segment positioned downstream from said promoter and operatively associated therewith, said promoter directing root cortex specific transcription of said heterologous DNA segment.

16. A transformed plant according to claim 15,  
20 wherein said root cortex specific promoter is a Tobacco RD2 promoter which directs root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

17. A transformed plant according to claim 15,  
25 wherein said promoter has a sequence selected from the group consisting of:

- (a) SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 provided herein, and  
30 (b) DNA sequences which hybridize to isolated DNA having a sequence of (a) above, under conditions represented by a wash stringency of 0.3M NaCl, 0.03 M

-44-

sodium citrate, 0.1% SDS at 60°, and which direct root cortex specific transcription of a downstream heterologous DNA segment in a plant cell.

18. A transformed plant according to claim 15,  
5 wherein said plant is a dicot.

19. A transformed plant according to claim 15,  
wherein said plant is a monocot.

20. a transformed plant according to claim 15,  
wherein said plant is a tobacco (*Nicotiana tabacum*)  
10 plant.

21. An isolated DNA molecule consisting  
essentially of a promoter which directs root cortex  
specific transcription of a downstream heterologous DNA  
segment in a plant cell and having a sequence selected  
15 from the group consisting of SEQ ID NOS:1-9 provided  
herein.

22. A DNA construct comprising an expression  
cassette, which construct comprises, in the 5' to 3'  
direction, a promoter according to claim 21 and a  
20 heterologous DNA segment positioned downstream from said  
promoter and operatively associated therewith.

23. A transformed plant comprising transformed  
plant cells, said transformed plant cells containing a  
DNA construct according to claim 22.

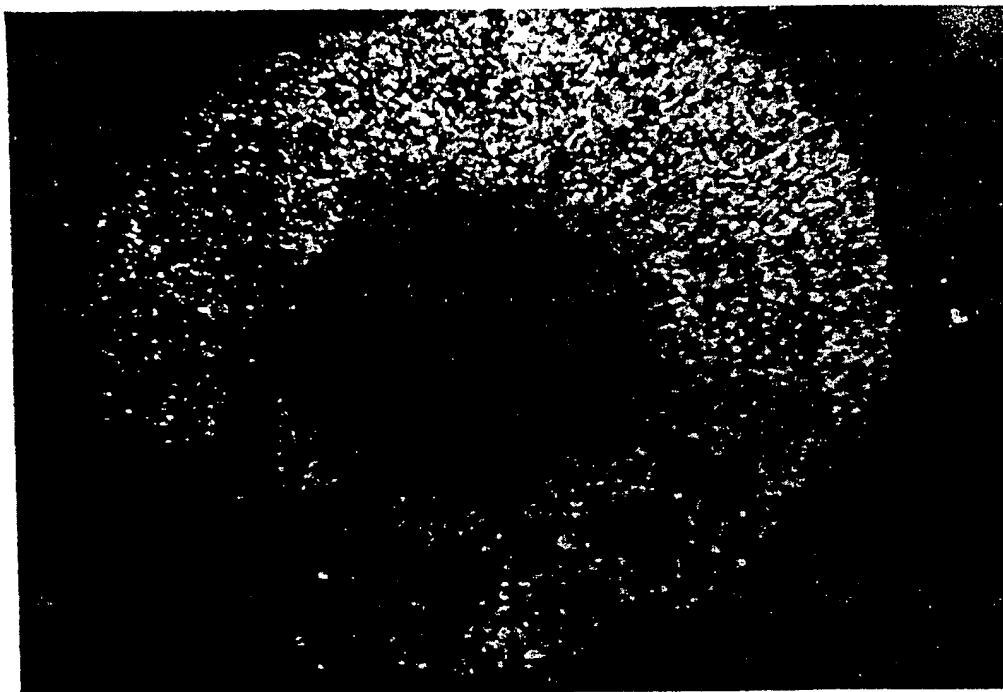


FIG. 1A.

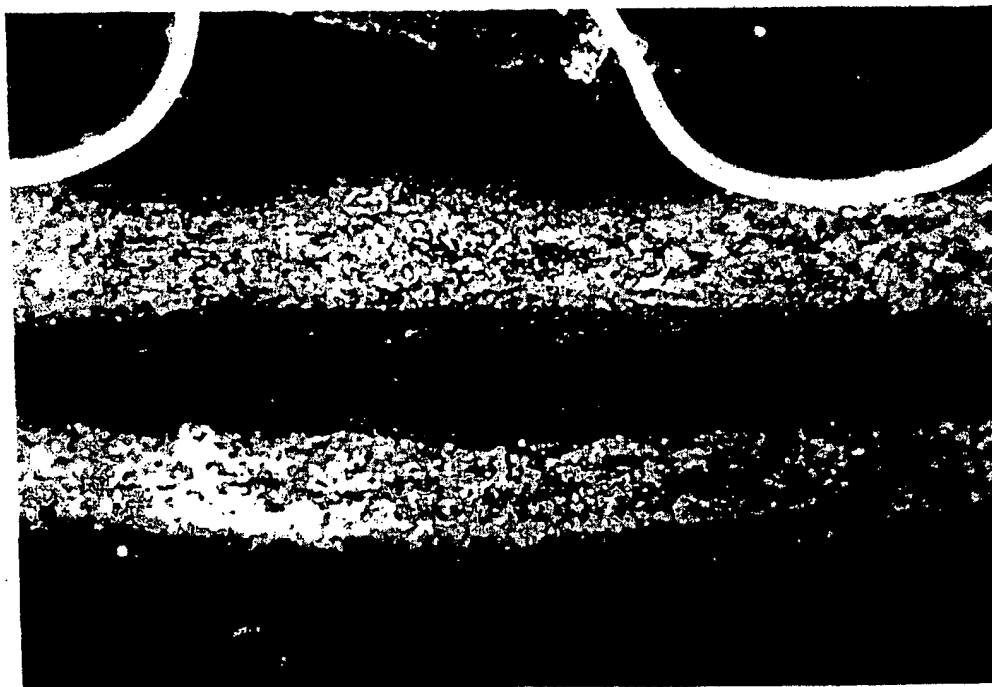


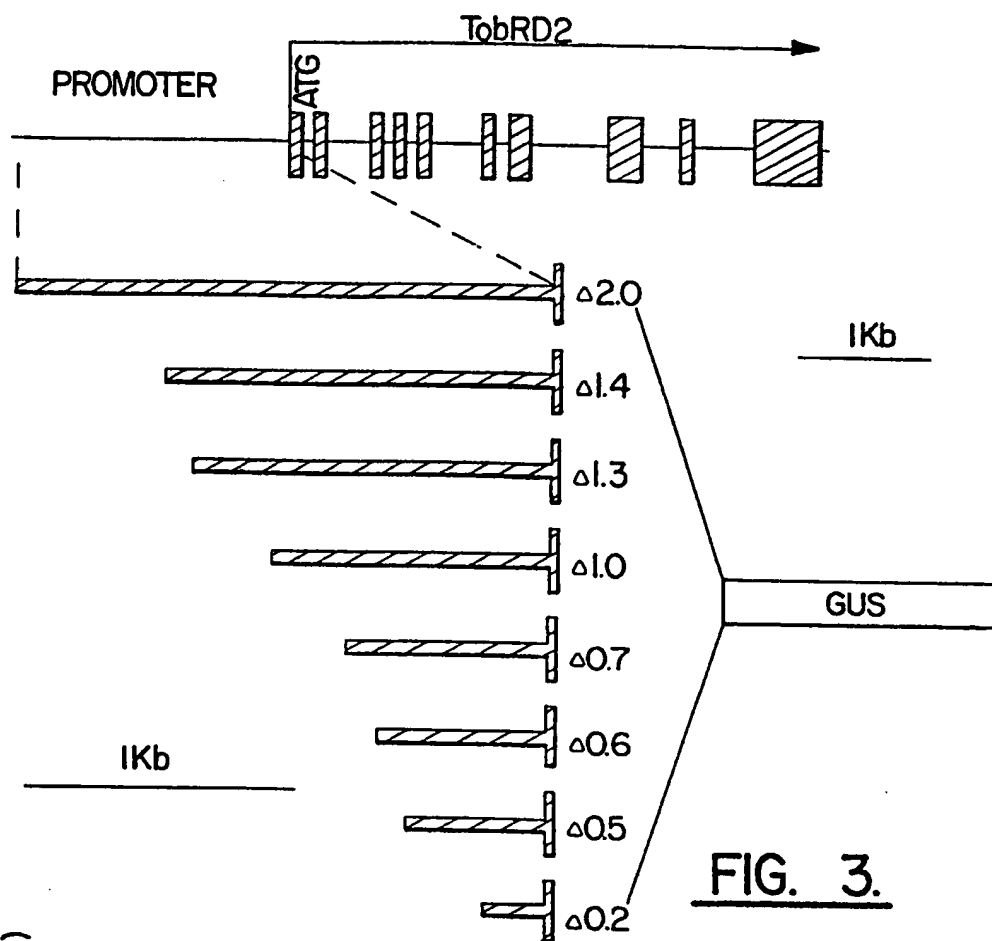
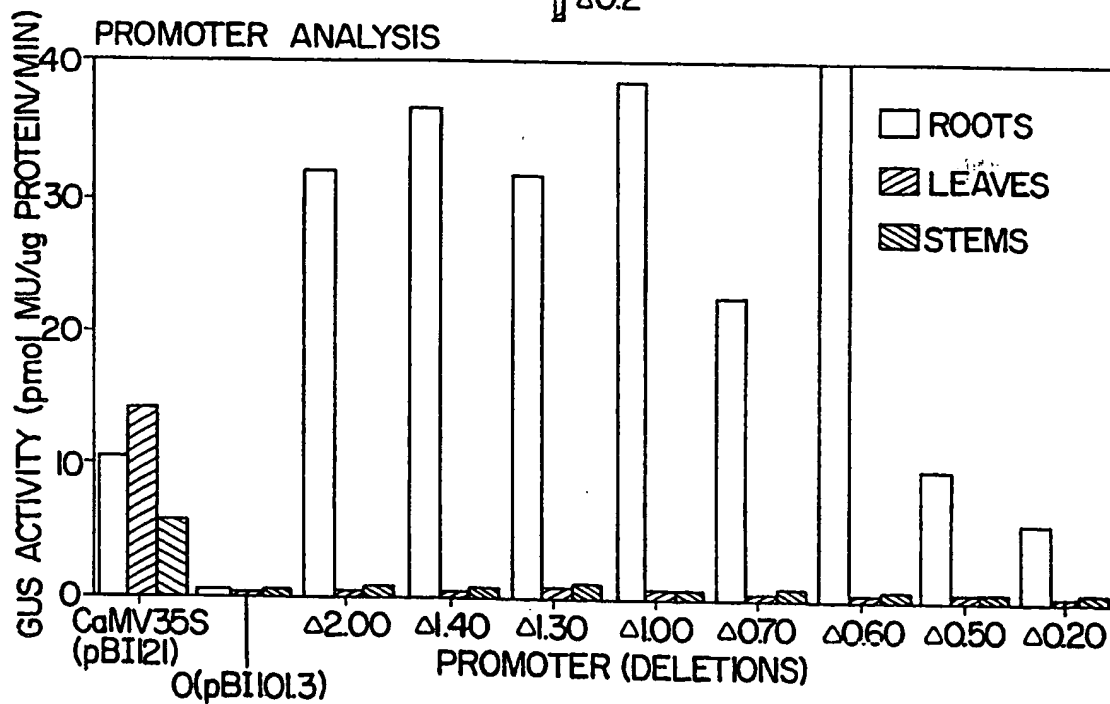
FIG. 1B.



CTCGAGGATC	TAAATTGTGA	GTTCAATCTC	TTCCCTATTG	GATTGATTAT	CCTTTCTTTT	60
CTTCCAATTT	GTGTTTCTTT	TTGCCTAATT	TATTGTGTTA	TCCCCTTTAT	CCTATTTTGT	120
TTCTTTACTT	ATTTATTTGC	TTCTATGTCT	TTGTACAAAG	ATTTAAACTC	TATGGCACAT	180
ATTTTAAAGT	TGTTAGAAAA	TAAATTCITT	CAAGATTGAT	GAAAGAACTT	TTTAAATTGA	240
GATATTTCTG	AGATTTTATT	CTCTTACTAC	CAATATAACG	CTTGAATTGA	CGAAAAATTG	300
TGTCCAAATA	TCTAGCAAAA	AGGTATCCAA	TGAAAAATA	TCATATGTGA	TCTTCAAATC	360
TTGTGTCTTA	TGCAAGATTG	ATACTTTGTT	CAATGGAAGA	GATTGTGTGC	ATATTTTAA	420
AATTTTTATT	AGTAATAAAG	ATTCTATATA	GCTGTATAG	AGGGATAATT	TTACAAAGAA	480
CACTATAAAT	ATGATTGTTG	TTGTTAGGGT	GTCAATGGTT	CGGTTCGACT	GGTTATTTTA	540
TAAAATTIGT	ACCATACCAT	TTTTTTCGAT	ATTCTATTTT	GTATAACCAA	AATTAGACTT	600
TTCGAAATCG	TCCCAATCAT	GTCGGTTTCA	CTTCGGTATC	GGTACCGTTC	GGTTAATTTT	660
CATTTTTTTT	TAAATGTCAT	TAAAATTCAC	TAGTAAAAAT	AGAATGCAAT	AACATACGTT	720
CTTTTATAGG	ACTTAGCAAA	AGCTCTCTAG	ACATTTTAC	TGTTTAAAGG	ATAATGAATT	780
AAAAAACATG	AAAGATGGCT	AGAGTATAGA	TACACAACCTA	TTCGACAGCA	ACGTAAAAAG	840
AACCAAGTAA	AAGCAAAGAA	AATATAAATC	ACACGAGTGG	AAAGATATTA	ACCAAGTTGG	900
GATTCAAGAA	TAAAGTCTAT	ATTAAATATT	CAAAAAGATA	AATTTAAATA	ATATGAAAGG	960
AAACATATTC	AATACATTGT	AGTTTGCTAC	TCATAATCGC	TAGAATACTT	TGTGCCTTGC	1020
TAATAAAGAT	ACTTGAAATA	GCTTAGTTTA	AATATAAATA	GCATAATAGA	TTTTAGGAAT	1080
TAGTATTTTG	AGTTTAATTA	CTTATTGACT	TGTAACAGTT	TTTATAATTC	CAAGGCCCAT	1140
GAAAAATTTA	ATGCTTTATT	AGTTTTAAAC	TTACTATATA	AATTTTTCAT	ATGTAAAATT	1200
TAATCGGTAT	AGTTTCGATAT	TTTTTCAATT	TATTTTATA	AAATAAAAAA	CTTACCCTAA	1260
TTATCGGTAC	AGTTATAGAT	TTATATAAAA	ATCTACGGTT	CTTCAGAAAG	AACCTAAAAA	1320
TCCGTTCCGT	GCGGACGGTT	CGATCGGTTT	AGTCGATTTT	CAAATATTCA	TTGACACTCC	1380
TAGTTGTGT	TATAGGTAAA	AAGCAGTTAC	AGAGAGGTAA	AATATAACTT	AAAAAATCAG	1440
TTCTAAGGAA	AAATTGACTT	TTATAGTAAA	TGACTGTTAT	ATAAGGATGT	TGTTACAGAG	1500
AGGTATGAGT	GTAGTTGGTA	AATTATGTTT	TTGACGGTGT	ATGTCACATA	TTATTTATTA	1560
AAACTAGAAA	AAACAGCGTC	AAAACTAGCA	AAAATCCAAC	GGACAAAAAA	ATCGGCTGAA	1620
TTTGATTGG	TTCCAACATT	TAAAAAAGTT	TCAGTGAGAA	AGAATCGGTG	ACTGTTGATG	1680
ATATAAACAA	AGGGCACATT	GGTCAATAAC	CATAAAAAAT	TATATGACAG	CTACAGTTGG	1740
TAGCATGTGC	TCAGTATTG	AACAAATCTA	AAGAAGGTAC	ATCTGTAACC	GGAACACCAC	1800
TTAAATGACT	AAATTACCCT	CATCAGAAAG	CAGATGGAGT	GCTACAAATA	ACACACTATT	1860
CAACAACCAT	AAATAAAACG	TGTTCAAGCTA	CTAAACAAA	TATAAATAAA	TCTATGTTTG	1920
TAAGCACTCC	AGCCATGTTA	ATGGAGTGCT	ATTGCCTGTT	AACCTCTACT	TATAAAATAG	1980
TAGTAGAAAA	AATATGAACC	AAAACACAAC				2010

FIG. 2.

3/6

**FIG. 3.****FIG. 4.**

PROMOTER ANALYSIS  
ROOTS/LEAVES

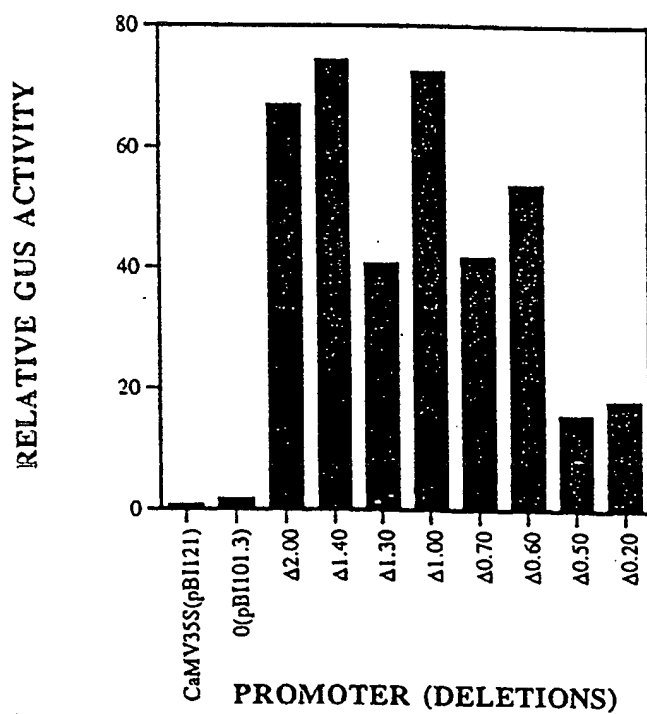


Fig. 5A

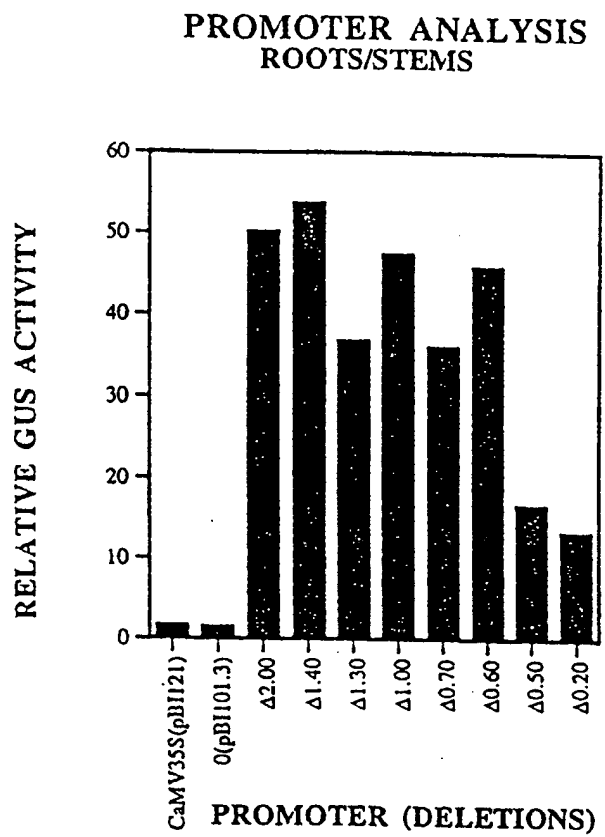


Fig. 5B

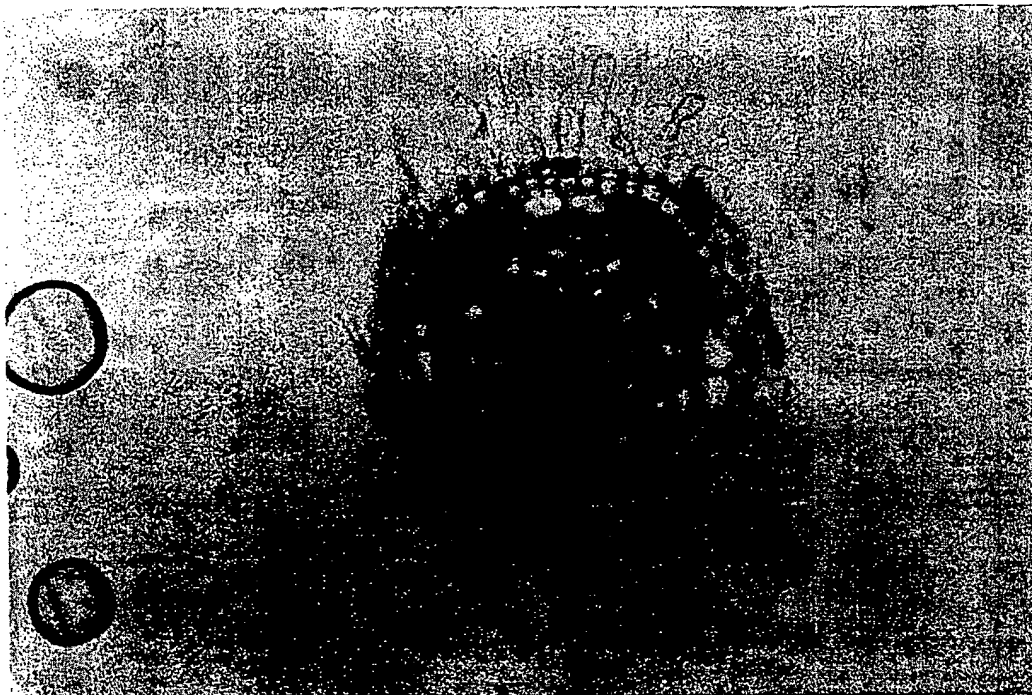


FIG. 6A.

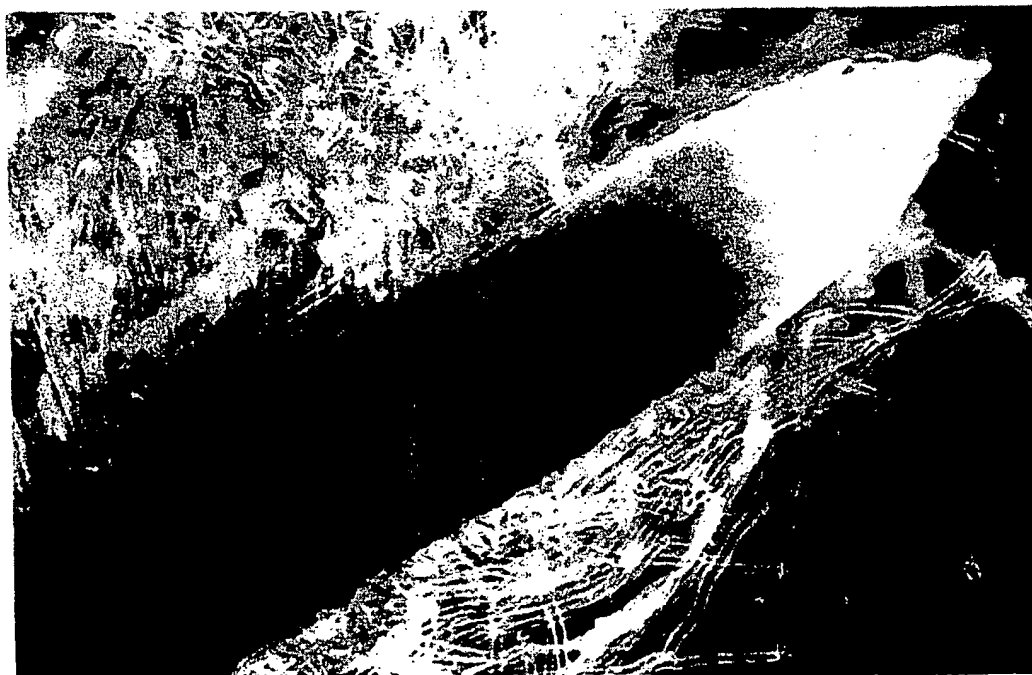


FIG. 6B.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/12158

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205, DIG 43; 536/24.1, 23.6, 23.71; 435/320.1, 252.2, 240.4, 240.47, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CABA, CAPLUS

search terms: root cortex, RD2, promoter, tissue specific, tobacco, expression

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CONKLING et al. Isolation of Transcriptionally Regulated Root-Specific Genes from Tobacco. Plant Physiology. 1990, Vol. 93, pages 1203-1211, especially page 1203.	1-23
Y	YAMAMOTO et al. Root-specific genes from tobacco and Arabidopsis homologous to an evolutionarily conserved gene family of membrane channel proteins. Nucleic Acids Research. 1990, Vol. 18, No. 24, page 7449.	1-23
X --- Y	US 5,097,025 A (BENFEY ET AL.) 17 March 1992, column 4, lines 5-68, column 5, column 6, lines 1-51.	15 ----- 1-23
Y	US 4,943,674 A (HOUCK ET AL.) 24 July 1990, column 1, lines 11-49.	1-23

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

05 SEPTEMBER 1996

Date of mailing of the international search report

24 OCT 1996

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Thomas Haas

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/12158

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 15/82, 5/10, 5/14, 15/00, 15/09, 15/29, 15/32; A01H 1/00, 5/00

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

800/205, DIG 43; 536/24.1, 23.6, 23.71; 435/320.1, 240.4, 172.3, 252.2, 240.47